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(54) Title: ANTIGENIC PREPARATIONS THAT STIMULATE PRODUCTION OF ANTIBODIES WHICH BIND TO THE PILI OF TYPE IV PILIATED BACTERIA

(57) Abstract

Antigenic preparations active against Type IV piliated bacteria comprise submolecular units of pilin protein. The submolecular units correspond to at least one epitope common to structural pilin proteins of Type IV piliated bacteria. The ability of such submolecular units to produce antibodies capable of binding to the whole pili can provide the basis for vaccines.

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ANTIGENIC PREPARATIONS THAT STIMULATE PRODUCTION OF ANTIBODIES WHICH BIND TO THE PILI OF TYPE IV PILIATED BACTERIA

FIELD OF THE INVENTION

The present invention relates to an antigenic preparation, capable of generating in vertebrates antibodies which bind to the whole pili of species of Type IV piliated bacteria. A specific embodiment of this invention relates to antigenic preparations active against *Bacteroides nodosus*. The antigenic preparations use submolecular units of *B. nodosus* pilin to elicit antibodies capable of blocking the pili function of *B. nodosus*. This pathogen is the essential causative agent of footrot infection in sheep and other ruminates.

BACKGROUND OF THE INVENTION

Pili are virulence factors for a wide range of bacteria pathogenic to both animal and humans. These pili have multiple functions that include epithelial cell adherence, microcolonization, adherence to other bacteria, twitching motility, and possibly other yet unexplored functions such as proteolytic enzyme or toxin delivery to target tissues. The pili of several genera of these including *Bacteroides (Porphyromonas), Moraxella, Pseudomonas, Vibrio,* pathogenic *E.Coli*, and *Neisseria* are unipolar and have an amino terminus methionine (*Vibrio* and some pathogenic *E.Coli*) or phenylalanine which is methylated (NMePhe) or lacking this are otherwise called Type IV pili. All Type IV pili share much sequence homology not only between strains within each bacterial species but between the different genera particularly in the first one third of the molecule (amino end). This segment (the first 1/3 of the

amino terminal end) is predominantly hydrophobic and seemingly less active biologically than the more antigenically variable remainder of the molecule.

One species of Type IV pilin bacteria that has undergone extensive study is *B. nodosus*. *B. nodosus* is the primary pathogen of sheep footrot. This agent can colonize the feet of sheep, produce proteases which progressively lyse layers of hoof and expose the underlying soft tissues to soil borne secondary infection. For *B. nodosus* to be pathogenic two virulence factors must be present. The organism must have pili and must produce proteases. Included in the proteases of virulent *B. nodosus* are enzymes that can hydrolyze elastin, collagen type 111, keratin, and other proteins. The pili or fimbria of pathogenic organisms in general are understood to function as organelles of adherence which bind the agent to appropriate host tissue or other organisms. Sometimes they exhibit a secondary functional characteristic of causing gliding or twitching motility. This later phenomena might simply represent release of mechanical forces that build up as the pili extrude from the cell, thus causing the cell to suddenly or gradually move a short distance. Although this motility may not contribute significantly to virulence, pili are thought to be a major, or perhaps the only, mechanism capable of effectively attaching the bacteria to sheep's feet and colonizing host tissue.

The pili antigens have been shown to be the protective antigens since antibodies against such pili can prevent sheep footrot (Stewart, D.J. (1978) **Res. Vet. Sci.** 24:14-19; Emery, D.L. et al. (1984) **Aust. Vet. J.** 61:237-238; Every, D. and Sherman, T.M. (1982) **New Z. Vet. J.** 30:156-158). This is also the case for *E. coli*, *Neisseria*, and other piliated pathogenic organisms where the pili are important as organelles of attachment (Schoolnik, G.K et al. (1983) **Prog. Aller.** 33:314-331;

Haggard, D.L. et al. (1982) **Vet. Med. Small Anim. Clin.** 77:1391-1394; Beachey, E.H. (1981) **J. Infect. Dis.** 143:325-345; Issacson, R.E. et al. (1978) **Infect. Immun.** 21:392-397; Salit, J.E. and Morgan, G. (1981) **Infect Immun.** 31:430-435). The serotype specificity of *B. nodosus* is shown to be dependent upon the antigenic determinants found on the pili (Every, D. (1979) **J. Gen. Microbiol.** 115:309-316; Egerton, J.R. (1973) **J. Comp. Path.** 83:151-159; Stewart, D.J. (1978) **Res. Vet. Sci.** 24:293-299). *B. nodosus* has been shown to carry some cross-reactive minor antigenic determinants on the pili (Stewart, D.J. et al. (1985) **Aust. Vet. J.** 62:153-159). This is the basis for the minor cross protection observed in some vaccine trials using piliated *B. nodosus* bacterins. Furthermore, a recombinant *Pseudomonas aeroginosa* has been constructed which expresses pili for single serotypes of *B. nodosus* (Stewart, D.J. et al., (1985) **Aust. Vet. J.** 62:153-159; Elleman, T.C. et al. (1986) **J. Bacteriol.** 168:574-580), but each single serotype affords only minor cross protection. This is because there are many different serotypes (peptide configurations) of pili and antibodies against one does not reliably or very often confer solid protection against the others.

The current commercial vaccines for *B. nodosus* are made up of whole bacterial cells including their pili each grown as a discrete serotype, (8 serotypes including 2 additional pilin protein variants of one of these type), which are then combined into a single vaccine. However, the efficacy of these polyvalent vaccines ranges from zero to 80% depending on how well the vaccine strains duplicate those strains which are actually infecting the sheep. In addition to such marginal efficacy, the current commercial vaccines use harsh adjuvants to drive up the antibody levels. These adjuvants cause severe tissue reactions sometimes resulting in abcess formation at inoculation sites. The

polyvalent vaccines currently being marketed stimulate production of a wide array of poorly targeted antibodies and many of these are of little or no use in conferring immunity. In other words, the sheep's immune reserves are squandered generating inappropriate or useless antibodies.

Therefore, a need continues to exist for a vaccine that elicits the production of antibodies that bind to the whole pili of strains within bacterial species, such as the various serotypes of *B. nodosus*, or between bacterial species of the Type IV pili class. Such a vaccine would perturb those pili functions conferring virulence and thereby, provide resistance to pathogens of the Type IV pili class. The present invention provides antigenic preparations to produce just such a vaccine using highly conserved antigenic segments of the Type IV pili class.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an antigenic preparation active against a species of Type IV piliated bacteria. The antigenic preparation comprises a submolecular unit of pilin protein corresponding to at least one epitope common to structural pilin proteins of the species of Type IV piliated bacteria. The submolecular unit of pilin protein is capable of eliciting antibodies capable of binding to the whole pili of the species of Type IV piliated bacteria. This ability to produce such antibodies provides the basis for effective vaccines against species of Type IV piliated bacteria. Antigenic preparations of the present invention can be prepared against Type IV piliated bacteria species such as *Bacteroides nodosus, Neisseria gonorrhea, Neisseria meningitis, Moraxella bovis, Vibrio cholera, Escherichia coli, and Pseudomonas aeroginosa*.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Bacteroides nodosus* is selected from the group of polypeptides consisting of:

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu;

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly Lys Tyr Ala Leu Ala;

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly Lys Tyr Ala Leu Ala Thr Ile Asp Gly Asp; Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser
Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met Arg Thr
Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly Lys Asp
Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu; and
Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly
Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile
Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln
Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu
Gly Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu Leu
Cys Ser Thr Asp Val Asp Glu Lys Phe Lys Pro Thr.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Neisseria gonorrhea* has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Val Gly
Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr Thr
Ala Arg Ala Gln Val Ser Glu Ala Ile Leu Leu Ala Glu Gly
Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Neisseria meningitis* has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Val Gly
Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr Thr
Ala Arg Ala Gln Val Ser Glu Ala Ile Leu Leu Ala Glu Gly
Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Moraxella bovis* has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Ile Gly
Ile Leu Ala Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr Ile
Ser Lys Ser Gln Thr Thr Arg Val Val Gly Glu Leu Ala Ala
Gly Lys Thr Ala Val Asp Ala Ala Leu Phe Glu Gly Lys Thr
Pro.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Vibrio cholera* has the following sequence:

Met Thr Leu Leu Glu Val Ile Ile Val Leu Gly Ile Met Gly Val Val Ser Ala Gly Val Val Thr Leu Ala Gln Arg Ala Ile Asp Ser Gln Asn Met Thr Lys Ala Ala Gln Ser Leu Asn Ser Ile Gln Val Ala Leu Thr Gln Thr.

The submolecular unit of pilin protein that is capable of eliciting antibodies against *Pseudomonas aeroginosa* has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Val Ala Arg Ser Glu Gly Ala Ser Ala Leu Ser Val Asn Pro Leu Lys Thr Thr Val Glu Glu Ala Leu Ser Arg Gly.

The invention further comprises an antigenic preparation of repeating sequences of polypeptides common to structural pilin proteins of the species of Type IV piliated bacteria.

The invention further comprises an antigenic preparation of at least one epitope of a polypeptide common to structural pilin proteins of the species of Type IV piliated bacteria.

The invention further comprises an antigenic preparation in which the submolecular unit of any part of the submolecular unit of pilin protein suspended in a suitable pharmaceutical carrier is used as a vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the immunoblot results of different *B. nodosus* serotypes versus a submolecular unit of pilin protein antibody;

FIG. 2 shows immunoelectron microscopy results for *B. nodosus* Type XV pili, one of the four known D-set pilin types, versus a submolecular unit of pilin protein antibody;

FIG. 3 shows immunoelectron microscopy results for *B. nodosus* A 198 pili, one of the 17 known A-set pilin Types, versus a submolecular unit of pilin protein antibody;

FIG. 4 shows a gene construct coding for a polypeptide of *B. nodosus*;

FIG. 5 shows a gene construct coding for a polypeptide of *B. nodosus*;

FIG. 6 shows a gene construct coding for a polypeptide of *B. nodosus*;

FIG. 7 shows a gene construct coding for a polypeptide of *B. nodosus*;

FIG. 8 shows a gene construct coding for a polypeptide of *B. nodosus*;

FIG. 9 shows a gene construct coding for a polypeptide of *N. gonorrhea*;

FIG. 10 shows a gene construct coding for a polypeptide of *N. meningitis*;

FIG. 11 shows a gene construct coding for a polypeptide of *M. bovis*;

FIG. 12 shows a gene construct coding for a polypeptide of *V. cholera*; and

FIG. 13 shows a gene construct coding for a polypeptide of *P. aeroginosa*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to antigenic preparations that produce antibodies that indirectly block or sterically interfere with pili function of pathogens having Type IV pili. Vaccines incorporating these antigenic preparations can provide protection against diseases caused by these pathogens. The approach of the present invention is based on finding a highly conserved antigenic segment, a submolecular unit of the Type IV pilin molecule, which will elicit the production of such antibodies. These antibodies bind to the whole pili of strains within bacterial species or between bacterial species. The result is that the antibodies perturb those pili functions conferring virulence and thereby provide resistance to pathogens of the Type IV pili class.

Finding such highly conserved antigenic segments is greatly aided by the following. First, the Type IV pili are made up exclusively or almost exclusively of a structural protein which is a polymerized repeat of a single molecular species. Second, the amino acid sequence and tertiary configuration of this molecule is one basis for the antigenic serotyping of pathogens having Type IV pili. Third, using *B. nodosus* as a modeling system, many serotypes (17 A-set pilin types of 21 *B. nodosus* described to date) bind to a single monoclonal antibody. Also the remaining four serotypes (D-set pilin types) bind with one other monoclonal antibody. Fourth, the antigens of the structural protein above are present in far greater numbers (perhaps 1000:1 up to 10,000:1) than any specific adhesion antigen associated with pili. Specific adhesion antigens are amino acid sequences presumably located on the tips or at intervals along the pili.

Using *B. Nodosus* as a model, highly conserved antigenic domains on the pilin protein were identified, isolated,

and then amplified as immunogens according to the following three steps. First, the conserved antigenic domains on the pilin protein molecule were determined. Second, the polypeptide sequence of these as subunits of the intact pilin protein were reproduced. Third, these subunits were tested as antigenic determinants for stimulating cross reactive antipilus antibodies.

The following are detailed procedures for carrying out the above three steps to determine submolecular units of pilin proteins capable of eliciting protective antibodies against Type IV pilin bacteria. The first step of selecting an antigenic site was accomplished according to the following three procedures. First, computerized predictions of the antigenic profile for known *B. nodosus* base sequences were generated. Second, pilin proteins were digested and then tested against a battery of monoclonal antibodies. Third, sequence homology was compared based on published sequences.

ANTIGENIC PROFILE PREDICTIONS

For antigenic profile predictions, computer generated tertiary configurations of pilin molecules were used. This computer program is based on the composite value of the five parameters of hydrophilicity, alpha helix, beta sheet, random coil, and beta turns and their potential as available antigen sites on any selected region of the pilin polypeptide.

PILIN PROTEIN DIGESTION

A number of enzymatic procedures were used to cleave the 151 AA sequences of *B. nodosus* pilin into specific fragments for testing as conserved epitopes (Smyth, Methods in Enzymology, Vol. XI: ed. by C.H.W. Hirs, Academic Press, N.Y., pp. 214-230, 1967; Jacobson et al., **J. Biol. Chem.** 248:6583-6591, 1973). The cited methods

were modified and trypsin digestion was completed after succinylation of lysine residues so that the pilin protein was cleaved on the carboxyl side of arginine residues to produce a peptide of approximately 5000 MW. This digest fragment contains one common epitope shared between 17 serotypes and is bound by the same monoclonal antibody which blocks adherence. A monoclonal antibody was used for demonstrating common antigens following the techniques described below. Adult BALB/c mice (Simonsen Laboratories, Gilroy, California) were injected intraperitoneally with purified pili (100 μg) that have undergone 4 cycles of $MgCl_2$ precipitation and an SDS-PAGE analysis. Three days before fusion (2-7 weeks after the initial injection), the mice were boosted with 20 μlg of pili intravenously. Spleen cells from each mouse were harvested, washed with serum-free media, and fused with SP2/0 myeloma cells in 50% polyethylene glycol. Fused cells were seeded into Linbro 96 well plates at 106 cells per well. Cells were fed with RPMI 1640 (Flow Laboratories) containing 15% HyClone defined fetal bovine serum and 1 mg/100 ml gentamicin and HAT. Hybridoma supernatants were screened for antibody production using ELISA. These procedures resulted in production of a family of monoclonals. One of these reacts with whole pili of 17 serotypes of *B. nodosus*, with purified pilin protein of these same serotypes, with a 5,000 MW fragment of pilin protein digest, and blocks attachment of *B. nodosus* to epithelial cells.

PEPTIDE SEQUENCING COMPARISONS

Published amino acid sequence data for 8 serotypes of *B. nodosus* are available (Elleman (1988) **Microbiol. Rev.** 52:233-247). Comparisons of these revealed areas of homology between all 8 serotypes. These areas were further examined for their antigenicity.

The second step of reproducing selected sequences was accomplished according to the following procedures. Selected peptides were synthesized. Then portions of *B. nodosus* pilin genome were amplified.

SYNTHETIC PEPTIDE ANALYSIS METHODS

The pilin protein of eight serotypes of *B. nodosus* have been sequenced and compared for homology. Using the methods of Chou and Fasman (**Ann Rev Biochem.** 47:251-276, 1978), the secondary structures represented by probable beta-turns were predicted. Also using computer generated models, three of these were compared for regions of hydrophobicity/hydrophilicity of the pilin. Using this rationale two peptides were synthesized where homology occurs between the pilin protein of various *B. nodosus* Australian strains. These were bound to carrier molecules (KLH) and used in rabbits to produce antibodies against the peptides. Although these antibodies did bind to the synthetic peptides, they bound poorly to whole pili and did not block pili adherence. Thus, these two regions were shown not to be of major interest as antigenic sites and focused attention on more highly conserved regions.

B. NODOSUS PILIN GENOME AMPLIFICATION

An Applied Biosystems Model 380A Synthesizer was used to synthesize oligonucleotides up to 50 bases in length. These oligonucleotides correspond to the entire primary structural gene that codes for the pilin of *B. nodosus* A198 incorporating phosphoramidites and standard methods. Also synthesized were complementary sequences to be used as bridges for reconstructing any portion of the genomic code for A198 pilin. Gaps in the second strand can be completed and sealed as desired using DNA polymerase I and DNA ligase. Using this technology, a specified

oligonucleotide of 153 bases was assembled. This gene can be amplified using a cloning vector.

In an alternate and more reliable approach PCR was used to amplify the desired genomic segments out of native *B. nodosus* cultures. This was accomplished by synthesizing two primers. The first primer was 27 bases with a Bam HI restriction site in the overhang as shown on the gene construct of Fig. 4. The second primer was 30 bases with a stop codon and Hind III site in the overhang as shown in the gene construct of Fig. 4. Such primer construction gave in-frame and directional efficiency for cloning. The primers were purified by acrylamide gel electrophoresis to give 2.5 mg/ml and 40 mg/ml, respectively. PCR amplification was accomplished with 25 cycles at 50°C annealing temperature. The resultant very tight band of *B. nodosus* DNA was purified by cTAB precipitation in high salt and 3 ammonium acetate precipitations with ethanol, giving a final DNA concentration of 500 ng/ul. The DNA fragment included the partial gene for the pilin protein molecule, and 21 additional bases including a stop codon. This PCR fragment insert was cloned into the over expression vector pTTQ8 (Amersham Cat. No. RPN 1259) and three of these clones were sequenced as follows. Inserts were primed with the m13/pUC forward sequencing primer using a sequence USB.X This primer matches the pTTQ8 vector at 5 bases downstream from the Hind III site on the 3′ side of the pTTQ8 polylinker and allowed direct sequencing of the Bam H1 through Hind III insert in the pTTQ8 plasmid. All three clones sequenced were the same 160 base fragment, all have an open reading frame from Bam H1 to Hind III, and were of the intended base sequence and number. To insure sufficient antigenicity for the small molecular weight peptide (< 10,000 daltons), the small peptide was expressed as a TrpE fusion protein. This was accomplished by subcloning into the pATH3 vector. The pATH3 system expressed a TrpE fusion protein of

approximately 40,000 daltons comprising about 10% of total protein production. This system was scaled up giving approximately 50 mg of pilin-TrpE fusion protein that was purified over a preparative SDS-PAGE gel.

The third step of testing antigenic characteristics of peptides was accomplished according to the following procedures. Antibodies were produced against the peptides. These antibodies were tested for binding specificity to *B. nodosus* pili.

ANTIBODY PRODUCTION

Antibodies were generated by administering the fusion protein subcutaneously and intramuscularly into rabbits. This was done using 1 mg amounts contained in polyacrylamide gel and complete Freund's adjuvant after the methods of Rothbard et al., **J. Exp. Med.** 110:208-221, 1984.

ANTIBODY BINDING SPECIFICITY TO *B. NODOSUS* PILI

The immunoblot procedure used a nitrocellulose membrane to which whole *B. nodosus* pili are fixed. The nitrocellulose binding sites unoccupied by transferred protein were saturated by incubation with 3% gelatin TBS for 1 hour. The treated nitrocellulose was incubated with antiserum dilution of 1:500 in TBS + 1% with gelatin, then washed 4 times 2 x 10 minutes with TBS and 0.05% Tween 20 and 2 x 10 minutes with Tween-free TBS pH 7.5. Antibody bound protein was then visualized by incubating for 1 hour in secondary antibody solution (goat antirabbit) conjugated with horseradish peroxidase diluted 1:2000 with antibody buffer. Then it was washed 4 times as above and developed with horseradish peroxidase color development. Using these immunoblot procedures, polyvalent rabbit antiserum, which was made against highly purified whole pilin, also bound the pilin protein.

Rabbits were inoculated with the 6,270 dalton fusion protein subunit of the pilin molecule contained in polyacrylamide gel and Freund's adjuvant. A 1:250 dilution of serum from the rabbits was used against 3 serotypes of *B. nodosus*, a whole bacteria and purified pilin preparation. The antipilus antibody produced in the rabbits, receiving the fusion protein, was detected by immunoblot techniques. See Table 1.

Table 1 IMMUNOBLOT: Three Serotypes of *B. nodosus* Purified Pili vs. Antibody to a 6270 Molecular Weight Replicate of Partial Pilin Expressed as a Fusion Protein with TrpE.

	ANTISERUM
*	Rabbits were given booster injections
+ =	Positive
± =	Very weak positive
- =	No detectable reaction

FIG. 1 shows the immunoblot results of different *B. nodosus* serotypes versus a submolecular unit of pilin protein antibody. Lane 1 showed the antiserum of rabbit #684 at pre-injection. Lane 2 showed antipilus antibody being produced in rabbit #684 against *B. nodosus* serotypes of A-set and D-set pili 98 days after receiving the 6,270 dalton fusion protein. Lane 3 showed the antiserum of rabbit #685 at pre-injection. Lane 4 showed antipilus antibody being produced in rabbit #685 against *B. nodosus* serotypes of A-set and D-set pili 98 days

after receiving the 6,270 dalton fusion protein. The differences in reaction between the samples of *B. nodosus* pili serotypes shown in FIG. 1 reflect the differences in pili concentration. The A 198 (A-set pilin) shown in FIG. 1 represent different sample passages.

The immunoelectron microscopy procedures carried out were a modification of those described by Lindberg et al. (1987) **Nature** 328:84-87. Whole *B. nodosus* purified pili were allowed to sediment onto a formvar-coated copper grid for 10 minutes. Then they were reacted three minutes against a drop of 1/10 dilution of antibody preparation in RLA-buffer followed by gentle (five minute) washing using P-buffer. The grids treated with the final antibody were washed for five minutes with P-buffer. Next the grids were negatively stained with 1% sodium silicotungstate and examined under the transmission electron microscope to detect the structural relationships of the pili.

Slide agglutination tests were run using lyophilized cultures of Eugon agar grown *B. nodosus* in aqueous suspension to provide approximately 10^7 bacteria/ml. Drops of this preparation were mixed with test sera on a slide and observed by light microscopy for agglutination or aggregation of the whole bacteria.

The use of colloidal gold label to detect antibody binding to pili was carried out through the following steps:

1. *B. nodosus* culture for agar plate resuspended in double distilled water, vortexes briefly, and clarified by centrifugation @ 1,000 x g for 10 minutes;
2. 10ul drop of supernatant place on a Formvar - coated grid for 20 minutes in a moist chamber @ 37°C;

3. grid was blotted and washed two times with tris buffered saline containing 0.3% Tween-20;
4. 10 ul drop of a 1:200 dilution of serum (in TBS/0.3% Tween) was placed on the grid and incubated for 90 minutes in a moist chamber @ 37°C;
5. grid was blotted and washed three times with TBS/Tween;
6. 10 ul drop of a 1:100 dilution of anti-rabbit IgG gold conjugate (10nm) was placed on the grid and incubated for 120 minutes in a moist chamber @ 37°C; and
7. grid was blotted and washed five times with TBS/Tween, rinsed three times with distilled water, and stained with 1.3% phosphotungstic acid @pH 7.0; then examined with a transmission electron microscope.

An alternate method to show aggregation of serum-treated pili, rather than just attachment of gold to pili uses the following steps:

1. *B. nodosus* culture for agar plate resuspended in double distilled water, vortexes briefly, and clarified by centrifugation @ 1,000 x g for 10 minutes;
2. 10 ul drop of supernatant and 10ul drop of 1:100 serum (diluted in TBS/0.3% Tween) mixed together in microcentrifuge tube, and incubated for 90 minutes @ 37°C;
3. 10 ul drop of mixture from Step #2 was placed on a Formvar-coated grid for 20 minutes in a moist chamber @ 37°C;
4. grid was blotted and washed three times with TBS/Tween;
5. 10 ul drop of a 1:100 dilution of anti-rabbit IgG gold conjugate (10 nm) was placed on the grid and incubated for 120 minutes in a moist chamber @ 37°C; and

6. grid was blotted and washed five times with TBS/Tween, rinsed three times with distilled water, and stained with 1.3% phosphotungstic acid @pH 7.0; then examined with a transmission electron microscope.

FIG. 2 shows immunoelectron microscopy results for *B. nodosus* Type II pili, one of the four D-set pilin Types, versus a submolecular unit of pilin protein antibody generated in rabbits using a 10 nm colloidal gold label. In FIG. 2 the pili without antibody are 5-6 nm in diameter. Those pili coated with antibody are 10-15 nm in diameter and show configurational disruption because of antibody cross binding.

FIG. 3 shows immunoelectron microscopy results for *B. nodosus* A 198 pili, one of the 17 known A-set pilin Types, versus a submolecular unit of pilin protein antibody generated in rabbits using a 10 nm colloidal gold label. In FIG. 3 the pili without antibody are 5-6 nm in diameter and the pili coated antibody which are 10-15 nm in diameter show configurational disruption. In both FIGS. 2 and 3 the colloidal gold label is less than the amount of bound antibody because the labeling reaction was not run to completion.

Antibodies against the submolecular units of pilin proteins bind pili of antigenic groups which represent all currently known *B. nodosus* serotypes causing them to clump. Clumping, which can be shown to be caused by antibody binding to the structural pilin protein molecule, has the effect of reducing the availability of adhesion proteins for attaching *B. nodosus* to host tissue. Thus, an antibody directed to common epitopes on structural pilin proteins of *B. nodosus* can mechanically interfere with its adherence to host tissue. This same stearic interference can similarly perturb all pili functions.

Five separate and distinct gene configurations, coding for *B. nodosus* polypeptides of approximately 6000, 7500, 8150, 8500, and 9150 molecular weight, were determined. See the sequences in FIGS. 4, 5, 6, 7, and 8. Also five additional sequences, representing *Neisseria gonorrhea, N. meningitis, Moraxella bovis, Vibrio cholera*, and *Pseudomonas aeroginosa*, were determined. All ten of these are constructs which may or may not have the first amino acid (phenylalanine, usually methylated) included. Each construct then continues with specific sequences, cut sites and stop codons such that they can be moved between vector systems. Examples of vectors include, but are not limited to, *E. coli, Pseudomonas*, yeasts, poxviruses. herpesvirus, and irridivirus. In this way either live virus vaccines or purified protein vaccines could be assembled depending upon efficacy, cost, feasibility and need.

As shown in FIG. 4, the first of these constructs is 150 bases with Bam Hl and Hind III restriction sites added at the 5′ and 3′ ends, respectively. Also a stop codon is added at the 3′ end. The second construct, as shown in FIG. 5, is identical to the construct in FIG. 4 except that 33 bases are inserted in front of both the stop codon and Hind III restriction site at the 3′ end. In FIG. 6 the construct is identical to the one in FIG. 5 except for the 15 bases added. Although the construct in FIG. 7 is not a modification of those in FIGS. 5 and 6, it is similar. It is made up of 207 bases with Bam Hl and Hind III sites added on the 5′ and 3′ ends, respectively. Also a stop codon is placed on the 3′ end. The construct in FIG. 8 differs from the one in FIG. 7 with the addition of 15 bases. All of these constructs are designed to express products with an appropriate and predicted alpha helix for histocompatibility processing. Thus, they may act as stand alone antigens (singlets) or as repeating units of antigens (doublets, triplets). They are also designed to be expressed with fusion

proteins such as Trp E for increasing the size of the molecule carrying the desired epitopes. Furthermore, synthetic peptides representing all or any antigenic portion of these constructs could be combined with a molecular carrier and used as antigens to generate antipili antibodies.

The five constructs as shown in FIGS. 9, 10, 11, 12, and 13 include approximately 150-159 bases, the aforementioned restriction sites, and stop codons. These constructs represent the DNA sequences for *N. gonorrhea, N. meningitis, M. bovis, V. cholera,* and *P. aeroginosa.* See FIGS. 9, 10, 11, 12, and 13, respectively. These constructs are designed so they can function in the same manner as the *B. nodosus* prototype construct.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the spirit or scope of the invention.

CLAIMS

1. An antigenic preparation active against a species of Type IV piliated bacteria comprising a submolecular unit of pilin protein corresponding to at least one epitope common to structural pilin proteins of the species of Type IV piliated bacteria, which submolecular unit is capable of eliciting antibodies capable of binding to the whole pili of the species of Type IV piliated bacteria.

2. An antigenic preparation according to claim 1 in which the submolecular unit of pilin protein is derived from a species selected from the group consisting of: *Bacteroides nodosus, Neisseria gonorrhea, Neisseria meningitis, Moraxella bovis, Vibrio cholera, Escherichia coli, and Pseudomonas aeroginosa*.

3. An antigenic preparation according to claim 2 against *Bacteroides nodosus*, wherein the submolecular unit is selected from the group of polypeptides consisting of:

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu;

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly Lys Tyr Ala Leu Ala;

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly Lys Tyr Ala Leu Ala Thr Ile Asp Gly Asp; Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu; and

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu Leu Cys Ser Thr Asp Val Asp Glu Lys Phe Lys Pro Thr.

4. An antigenic preparation according to claim 3, further comprising repeating sequences of any of the polypeptides.

5. An antigenic preparation according to claim 3, further comprising at least one epitope of any of the polypeptides.

6. An antigenic preparation according to claim 2 against *Neisseria gonorrhea*, wherein the submolecular unit has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Val Gly Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr Thr Ala Arg Ala Gln Val Ser Glu Ala Ile Leu Leu Ala Glu Gly Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn.

7. An antigenic preparation according to claim 6, further comprising repeating sequences of the polypeptide.

8. An antigenic preparation according to claim 6, further comprising at least one epitope of the polypeptide.

9. An antigenic preparation according to claim 2 against *Neisseria meningitis*, wherein the submolecular unit has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Val Gly Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr Thr Ala Arg Ala Gln Val Ser Glu Ala Ile Leu Leu Ala Glu Gly Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn.

10. An antigenic preparation according to claim 9, further comprising repeating sequences of the polypeptide.

11. An antigenic preparation according to claim 9, further comprising at least one epitope of the polypeptide.

12. An antigenic preparation according to claim 2 against *Moraxella bovis*, wherein the submolecular unit has the following sequence:

Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr Ile Ser Lys Ser Gln Thr Thr Arg Val Val Gly Glu Leu Ala Ala Gly Lys Thr Ala Val Asp Ala Ala Leu Phe Glu Gly Lys Thr Pro.

13. An antigenic preparation according to claim 12, further comprising repeating sequences of the polypeptide.

14. An antigenic preparation according to claim 12, further comprising at least one epitope of the polypeptide.

15. An antigenic preparation according to claim 2 against *Vibrio cholera*, wherein the submolecular unit has the following structure:

Met Thr Leu Leu Glu Val Ile Ile Val Leu Gly Ile Met Gly Val Val Ser Ala Gly Val Val Thr Leu Ala Gln Arg Ala Ile Asp Ser Gln Asn Met Thr Lys Ala Ala Gln Ser Leu Asn Ser Ile Gln Val Ala Leu Thr Gln Thr.

16. An antigenic preparation according to claim 15, further comprising repeating sequences of the polypeptide.

17. An antigenic preparation according to claim 15, further comprising at least one epitope of the polypeptide.

18. An antigenic preparation according to claim 2 against *Pseudomonas aeroginosa*, wherein the submolecular unit has the following structure:

Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Val Ala Arg Ser Glu Gly Ala Ser Ala Leu Ser Val Asn Pro Leu Lys Thr Thr Val Glu Glu Ala Leu Ser Arg Gly.

19. An antigenic preparation according to claim 18, further comprising repeating sequences of the polypeptide.

20. An antigenic preparation according to claim 18, further comprising at least one epitope of the polypeptide.

21. An antigenic preparation as claimed in any one of claims 1 to 20 in which the submolecular unit or any part of the submolecular unit of pilin protein is suspended in a suitable pharmaceutical carrier and used as a vaccine.

22. An antigenic preparation as claimed in claim 21 which includes an adjuvant.

Sheet 1 of 13

Fig. 1

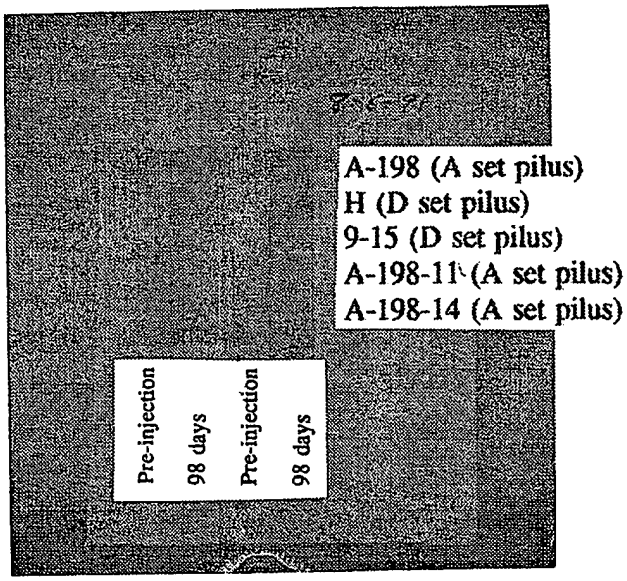


Fig. 2

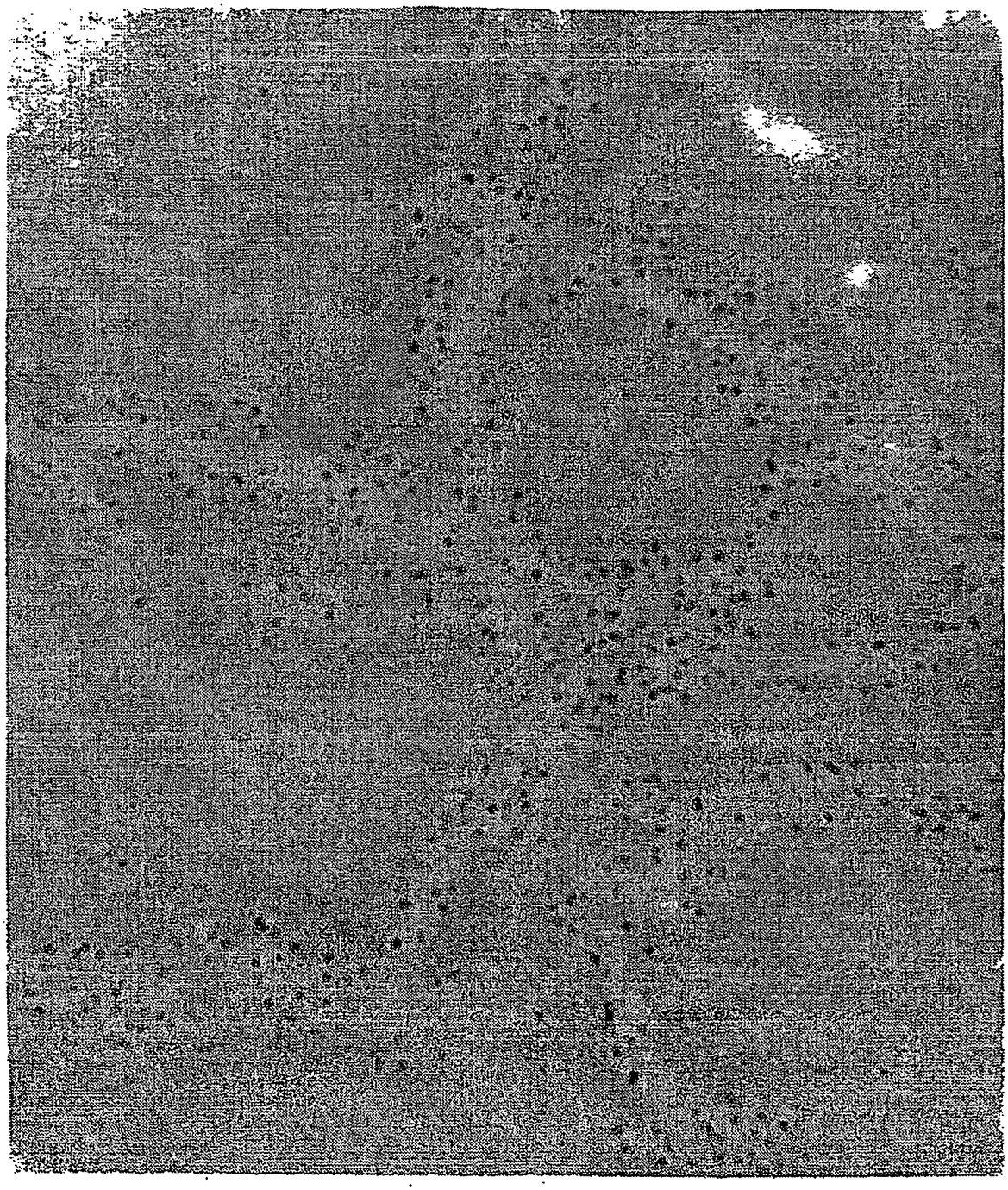


Fig. 3

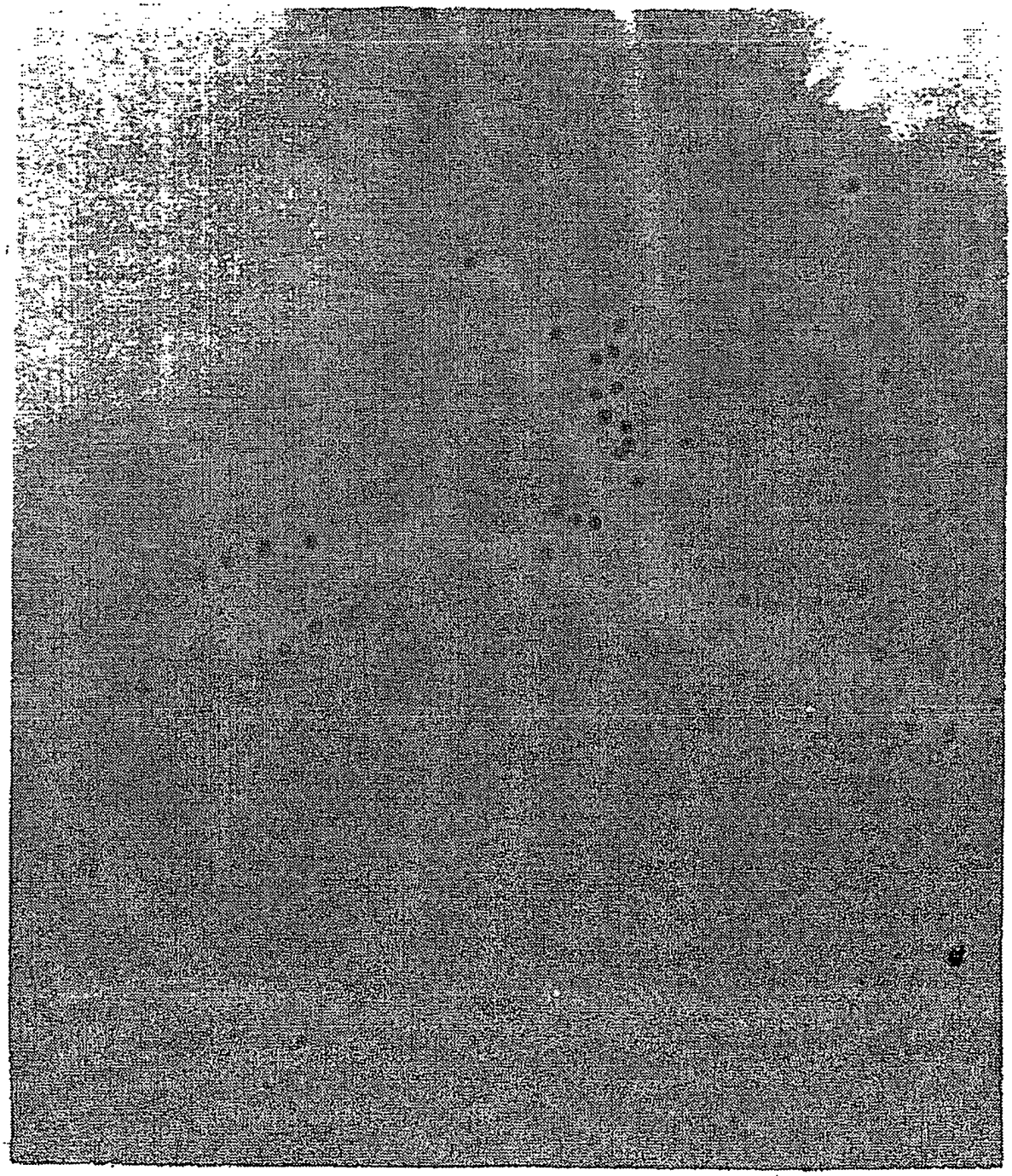


FIG. 4

Bam HI Site

5′ GCC GGA TCC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC GGT ATC TTA GCG
Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

GCT TTC GCT ATC CCT GCA TAT AAC GAC TAC ATC GCT CGT TCA CAA GCA GCT GAA GGC TTA
Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu

ACA TTG GCT GAT GGT TTG AAG GTT CGC ATT TCT GAT CAC TTA TAA GCT TCG AAG 3′
Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Hind III Site

Stop Codon

FIG. 5

```
        Bam HI
        SITE
5' GCC TGA TCC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC GGT ATC TTA GCG
               Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

   GCT TTC GCT ATC CCT GCA TAT AAC GAC TAC ATC GCT CGT TCA CAA GCA GCT GAA GGC TTA
   Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu

   ACA TTG GCT GAT GGT TTG AAG GTT CGC ATT TCT GAT CAC TTA GGT AAT GAT GAT AAA GGT
   Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly

   AAA TAC GCT CTT GCT TAA GCT TCG AAG 3'
   Lys Tyr Ala Leu Ala  Hind III
                       Site

                       ___
                       Stop
                       Codon
```

FIG. 6

```
      Bam HI
      Site
5'GCC GGA TCC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC GGT ATC TTA GCG
              Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

  GCT TTC GCT ATC CCT GCA TAT AAC GAC TAC ATC GCT CGT TCA CAA GCA GCT GAA GGC TTA
  Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala Ala Glu Gly Leu

  ACA TTG GCT GAT GGT TTG AAG GTT CGC ATT TCT GAT CAC TTA GGT AAT GAT GAT AAA GGT
  Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Gly Asn Asp Asp Lys Gly

  AAA TAC GCT CTT GCT ACA ATT GAT GGT GAT TAA GCT TCG AAG 3'
  Lys Tyr Ala Leu Ala Thr Ile Asp Gly Asp  Hind III
                                           Site

                                          Stop
                                          Codon
```

FIG. 7

Bam HI Site

5' GCC GGA TCC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC GGT ATC TTA GCT
Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

GCA ATC GCT ATT CCA CAA TAC CAA AAC TAC ATC GCT CGT TCA CAA GTT AGC CGC GTT ATG
Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met

TCA GAA ACT GGA CAA ATG CGC ACT GCC ATC GAA ACT TGC CTT TTA GAT GGT AAA GAA GGA
Ser Glu Thr Gly Gln Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly

AAA GAT TGC TTC ATT GGT TGG ACC ACA AGT AAC TTA TAA GCT TCG AAG 3'
Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu

Hind III Site

Stop Codon

FIG. 8

Bam HI Site

```
5′ GCC GGA TCC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC GGT ATC TTA GCT
            Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

   GCA ATC GCT ATT CCA CAA TAC CAA AAC TAC ATC GCT CGT TCA CAA GTT AGC CGC GTT ATG
   Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met

   TCA GAA ACT GGA CAA ATG CGC ACT GCC ATC GAA ACT TGC CTT TTA GAT GGT AAA GAA GGA
   Ser Glu Thr Gly Gln Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly

   AAA GAT TGC TTC ATT GGT TGG ACC ACA AGT AAC TTA TTA TGC TCA ACA GAC GTT GAT GAA
   Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu Leu Cys Ser Thr Asp Val Asp Glu

   AAA TTC AAG CCA ACT GGC TGT AAA TAA GCT TCG AAG 3′
   Lys Phe Lys Pro Thr Gly Cys Lys  Hind III
                                    Site
                                   ___
                                   Stop
                                   Codon
```

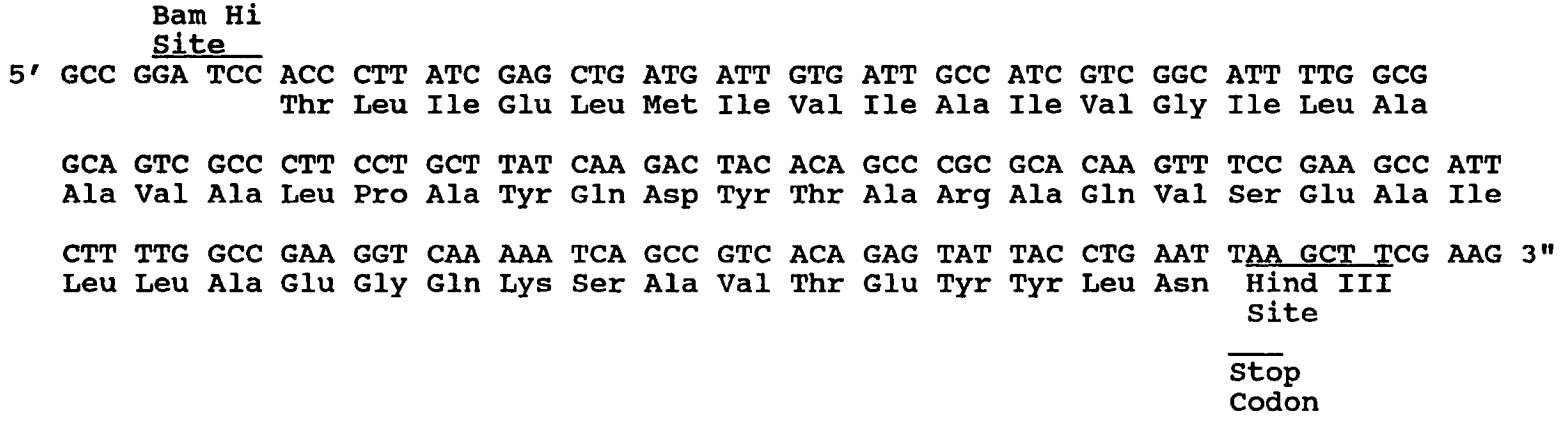
FIG. 9

```
                Bam HI
                Site
5' GCC GGA TCC ACC CTT ATC GAG CTG ATG ATT GTG ATC GCT ATC GTC GGC ATT TTG GCG
               Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Val Gly Ile Leu Ala

   GCA GTG GCC CTT CCC GCC TAC CAA GAC TAC ACC GCC CGC GCG CAA GTT TCC GAA GCC ATC
   Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr Thr Ala Arg Ala Gln Val Ser Glu Ala Ile

CTT TTG GCC GAA GGT CAA AAA TCA GCC GTC ACC GAG TAT TAC CTG AAT TAA GCT TCG AAG 3'
Leu Leu Ala Glu Gly Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn   Hind III
                                                                    Site
                                                                  ---
                                                                  Stop
                                                                  Codon
```

FIG. 10



SUBSTITUTE SHEET

FIG. 11

Bam HI Site

5' GCC GGA TCC ACC CTT ATC GAA TTG ATG ATT GTT ATC GCC ATT ATT GGT ATC CTA GCT
Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile Ile Gly Ile Leu Ala

GCA ATC GCT CTA CCT GCT TAC CAA GAC TAT ATC TCT AAG TCT CAA ACT ACT CGT GTA GTT
Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr Ile Ser Lys Ser Gln Thr Thr Arg Val Val

GGC GAA CTA GCT GCT GGT AAA ACT GCT GTG GAT GCT GCT TTG TTT GAG GGT AAA ACT CCC
Gly Glu Leu Ala Ala Gly Lys Thr Ala Val Asp Ala Ala Leu Phe Glu Gly Lys Thr Pro

TAA GCT TCG AAG 3'
Hind III Site

Stop Codon

FIG. 12

```
                Bam HI
                Site
5' GCC GGA TCC ATG ACA TTA CTC GAA GTG ATC ATC GTT CTA GGC ATT ATG GGG GTG GTT
               Met Thr Leu Leu Glu Val Ile Ile Val Leu Gly Ile Met Gly Val Val

   TCG GCG GGG GTT GTT ACT CTG GCG CAG CGT GCG ATT GAT TCG CAG AAT ATG ACC AAG GCC
   Ser Ala Gly Val Val Thr Leu Ala Gln Arg Ala Ile Asp Ser Gln Asn Met Thr Lys Ala

   GCG CAA AGT CTC AAT AGT ATC CAA GTT GCA CTG ACA CAG ACA TAA GCT TCG AAG 3'
   Ala Gln Ser Leu Asn Ser Ile Gln Val Ala Leu Thr Gln Thr  Hind III
                                                            Site
                                                           ---
                                                           Stop
                                                           Codon
```

FIG. 13

```
                Bam HI
                Site
5' GCC GGA TCC ACC TTG ATC GAA CTG ATG ATC GTG GTT GCG ATC ATC GGT ATC TTG GCT
               Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala

   GCA ATT GCC ATT CCT CAG TAT CAG AAT TAT GTA GCT CGT TCG GAA GGC GCA TCT GCT CTT
   Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Val Ala Arg Ser Glu Gly Ala Ser Ala Leu

   GCT TCG GTC AAT CCG TTG AAG ACT ACC GTT GAA GAG GCG CTT TCT CGT GGT TAA GCT TCG AAG 3'
   Ala Ser Val Asn Pro Leu Lys Thr Thr Val Glu Glu Ala Leu Ser Arg Gly   Hind III
                                                                          Site
                                                                    Stop
                                                                    Codon
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/11085

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/095, 39/106, 39/108, 39/104; C12N 1/00, 1/20
US CL :424/92; 435/7.3, 69.1, 252.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/92; 435/7.3, 69.1, 252.33, 849, 871, 875

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, CAS, Embase, Agricola, Life Sciences, Zoological Record, WPI, Pascal, A-GeneSeq 8, PIR, Swiss-Prot 23

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,622,223 (Schoolnik et al) 11 November 1986, col. 9, line 28.	1-20
Y	US, A, 4,737,363 (Stewart et al) 12 April 1988, col. 2, line 13.	21-22
Y	J.R. Egerton et al, "Footrot and foot abscess of ruminants" published 1989 by CRC Press, Inc. (Boca Raton, FL), pages 220-224, especially pages 225 and 230.	1-22
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 261, No. 33, issued 25 November 1986, K. Johnson et al, "Nucleotide Sequence and Transcriptional Site of Two Pseudomonas aeruginosa Pilin Genes", pages 15703-15708, especially page 15708.	18-20
A	GENE, Volume 85, No. 1, issued 1989, R. Faast et al, "Nucleotide sequence of the Structural Gene, tcp A, for a Major Pilin Subunit of Vibrio cholerae:, pages 227-231.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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18 FEBRUARY 1993

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INTERNATIONAL SEARCH REPORT

International applicanon No.
PCT/US92/11085

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 228, issued 24 May 1985, J.F. Young et al, "Expression of Plasmodium falciparum Circumsporozoite Proteins in Escherichia coli for Potential Use in a Human Malaria Vaccine", pages 958-962, especially page 962.	1-20
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 168, issued September 1988, W.W. Reuhl et al, "Purification, Characterization, and Pathogenicity of Moraxella bovis Pili", pages 983-1002, especially page 995.	12-14
Y	MOLECULAR MICROBIOLOGY, Volume 2, No. 5, issued September 1988, W.J. Potts et al, "Nucleotide Sequence of the Structural Gene for Class I Pilin from Niesseria meningitidis: Homologies with the pilE Locus of Neisseria gonorrhoeae", pages 647-653, especially page 651.	9-11

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